Investigating biomarkers in tear fluid for rapid diagnosis of diabetic retinopathy using single mode optical fiber biosensor

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DECLARATION

I hereby declare that this manuscript, entitled "Investigating biomarkers in tear fluid for rapid diagnosis of diabetic retinopathy using single mode fiber optics biosensor" is the result of my work except for the quotations and citations which have been duly acknowledged.

I also declare that, to the best of my knowledge and belief, it has not been previously submitted in whole or in part, for any other degree or diploma at Nazarbayev University or any other national or international institution.

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List of abbreviations

CO_2	Carbon dioxide
DM	Diabetes Mellitus
DR	Diabetic Retinopathy
ELISA	Enzyme-Linked Immunoassay
LCN-1	Lipocalin-1
LMIC	Low and Middle-Income Countries
LoD	Limit of Detection
NDR	Non-Diabetic Retinopathy
NPDR	Non-Proliferative Diabetic Retinopathy
OBR	Optical Backscatter Reflectometer
OFBR	Optical Fiber Ball Resonator
PDR	Proliferative Diabetic Retinopathy
RI	Refractive Index
SMF	Single Mode Fiber

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Abstract

Diabetic retinopathy is one of the most prevalent and the most distinct complication of diabetic mellitus, which is estimated to affect 700 million people globally by 2045. Previous studies have shown that about 14% of patients with type 1 and 33% of patients with type 2 diabetes progress to develop diabetic retinopathy. This shows the ultimate need for quick, effective, and affordable screening of diabetic retinopathy for early intervention.

Besides the high purchase cost, existing technologies used for screening diabetic retinopathy using tear fluid have registered significant challenges due to the limited volume accessible for analysis; the collection of tear fluid from patients; and the tedious laboratory processing for a timely diagnosis. Optical fiber biosensors have transformed biosensing platforms by bringing added benefits which include simplicity of fabrication and operation, low-cost, rapid response time, miniaturized size, high sensitivity and specificity, robustness, in-situ measurement capability, and can easily be interfaced for data analysis, making them ideal for a very wide range of applications.

The proposed study uses fiber optic biosensors as affinity biosensors to detect LCN-1 protein spiked in artificial tear fluid using a wearable eye-goggle tear fluid sampling device that facilitates the interaction of tear fluid sample with an optical fiber ball resonator (OFBR) biosensor. This study aims to prove this concept and eliminate the inconsistencies in existing sample collection, storage, and laborious processing methods currently being used in conventional tear fluid analysis. The experiments involved evaluating static and in-situ measurements of LCN-1 protein in artificial tears using a wearable sampling device. Results obtained from this experiment has demonstrated the potential of using the proposed concept for detecting biomarker in tear fluid using single-mode fiber optic biosensor.

CHAPTER 1: Introduction

1.1 Diabetic retinopathy

Diabetic retinopathy is one of the most common complications of diabetes mellitus and has been considered the leading cause of acquired vision loss in adults within their productive age in society [1]. The ambiguities in the pathogenesis of diabetic retinopathy make it difficult to achieve effective clinical treatment for this complication making early diagnostic and screening the only option available to mitigate the risk of blindness [2]. Previous studies implicated long-term diabetes, poor management of blood sugar and blood pressure level, and dyslipidemia among the risk factors driving DR development; but the main cause of DR has not been exclusively defined since other long-term diabetes patients with poorly managed glycemic control do not show signs of DR [3]. Several past studies have indicated that the risk of developing DR can be minimized by proper control of patient blood pressure and blood sugar levels [4]. Besides the effect DR has on eyesight, it also indicates an elevated risk of deadly underlying vascular problems [5].

According to the International Diabetic Federation (IDF) in one of the most recent publications, diabetic retinopathy is the most prevalent and the most distinct complication of diabetic mellitus, which is estimated to affect 700 million people globally by 2045 [6]. Previous studies have shown that about 14% of patients with type 1 and 33% of patients with type 2 diabetes progress to develop diabetic retinopathy [5]. This shows the ultimate need for quick, effective, and affordable screening of diabetic retinopathy for early intervention. The development of point-of-care devices for screening diabetic retinopathy biomarkers like LCN-1 will enable rapid reporting, enhances the effectiveness of patient treatment response with less challenge to the healthcare providers, and eliminate prolonged waiting for lab findings to decide on diagnosis [7].

1.2 Biomarker for diabetic retinopathy in tear fluid

Tear fluids provide the opportunity to non-invasively access and screen for biomarkers that predicates the development of diseases like diabetic retinopathy [8]. Biomarkers that can manifest in tear fluid consist of proteins, organic metabolites as well as electrolytes, and lipids. These can be a potential alternative to available screening methods for early diagnostics and can be easily integrated into routine healthcare monitoring [9]. Four key proteins are found in human tear fluid namely, lysozyme, lactoferrin, lipocalin 1, and secretory immunoglobin A. Lipocalin 1 (LCN-1) is one of the highly and significantly expressed proteins in tear fluid of

patients with diabetic retinopathy in an early stage [1]. Hence the ability to use a low cost, and low-cost diagnostic technique that can be integrated as routine clinical screening for LCN-1 biomarkers could be a game changer in abating blindness in patients with diabetes mellitus. This technique should overcome the current methods used for tear fluid sampling, processing, and analysis used in diabetic retinopathy screening.

1.3 Optic fiber biosensor

Fiber optic biosensors are gradually being developed into analytical biosensors devices that can be used to translate the biochemical concentration of an analyte into a measurable optical signal by interacting directly with the analyte. They are also used to measure the concentration of some analytes in very low concentrations. Optical fiber biosensors have transformed biosensing platforms by bringing added benefits which include simplicity of fabrication and operation, low-cost, rapid response time, miniaturized size, high sensitivity and specificity, robustness, in-situ measurement, and can easily be interfaced for data analysis, making them ideal for a very wide range of applications [10].

Existing technologies used for screening diabetic retinopathy in tear fluid have registered significant challenges due to limited tear fluid volume for analysis, collection of tear fluid from patients, and the tedious laboratory processing to come up with a timely diagnosis [11]. This study will use already transferable technologies to creatively integrate them into a point-of-care device for sampling and analysis of tear fluid to screen for LCN-1 biomarkers in tear fluid.

1.4 Aim of the study

The proposed study uses fiber optic biosensors as affinity biosensors to detect LCN-1 protein in spiked artificial tear fluid using a wearable eye-goggle tear fluid sampling device that facilitates the interaction of tear fluid sample with an optical fiber ball resonator (OFBR) biosensor. This study aims to prove the concept of using optical fiber biosensors to eliminate the inconsistencies in current existing sample collection, storage, and laborious processing currently being used in conventional tear fluid analysis by using low-cost and easily scalable label-free optical fiber biosensors. the fabrication process of this proposed concept will take advantage of the low-limit of detection ability of the optical fiber ball resonator achieve by A. Bekmurzayeva et al. in-situ use of OFBR biosensor.

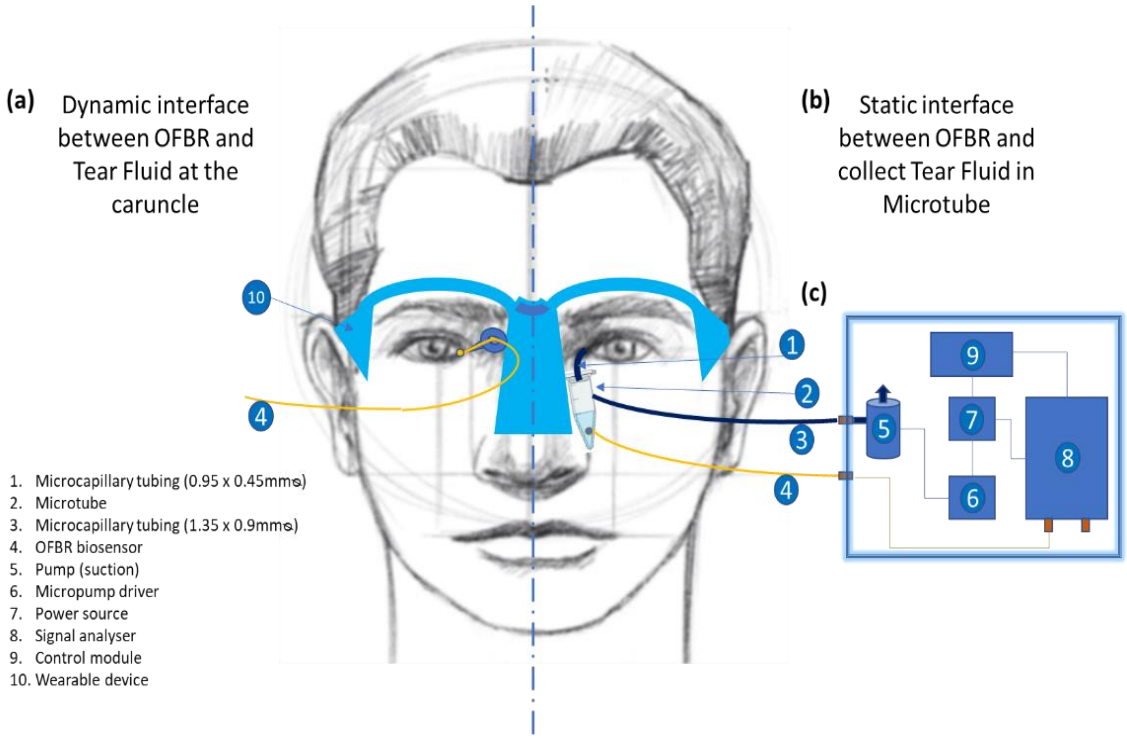


Figure 1. 1: Proposed concept of a wearable device for biomarker detection in tear fluid. (a) In-situ measurement in dynamic mode, (b) static measurement. (Facial sketch modified from Fussell, n.d.)

1.5 Literature review

Body fluids like a tear, blood, saliva, and sweat have attracted major research focus in the search for predictive biomarkers in both communicable and non-communicable diseases. Except for blood, the non-invasive access to tears, saliva, and sweat makes these body fluids a potential target to explore crucial biomarkers for predicting, diagnosing, and monitoring diseases [8]. Research trend shows the increasing need to investigate biomarkers for the predictive diagnosis of diseases, monitoring disease progression, and therapy.

The study of tear fluid is becoming an increasing area of interest due to the unique potential it possesses as a comprehensive body fluid that can be used for routine clinical diagnostics. Tear fluid has been considered a relevant source of clinical information relating to specific body parts through the screening of biomarkers [12]. Clinicians, especially in ophthalmology have developed a great interest in the exploration of tear fluid for potential biomarkers for a range of systemic eye diseases. Biomarkers found in tear fluids can serve as an effective alternative or enhancement to current techniques used for the diagnosis, monitoring, and predictive detection of diseases using fast and low-cost tear fluid analysis systems.

Tear fluid contains secretions from the lacrimal gland, goblet cells, cornea, and vascular tissues that keep the eye healthy. Tear fluid forms a film that consists of 3 main layers [13]: the mucin, the aqueous, and the lipid layer (Fig.1). The mucin layer is the lower

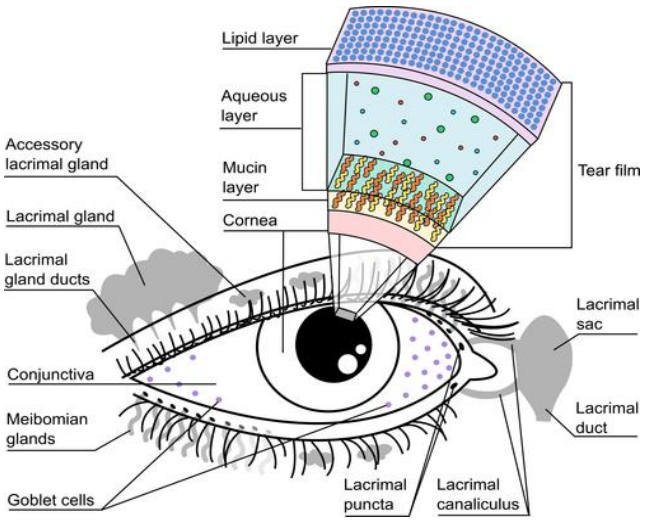


Figure 1. 2: Illustration of the surface structure and components of the eye [13].

hydrophilic mucous layer produced by the conjunctival goblet cell that maintains the aqueous layer on the surface of the eyeball [14]. The lipid layer is produced by the meibomian glands towards the outer margin of the eyelid as well as in the aqueous layer. The tear fluid produced by the lacrimal gland constitutes various proteins and antibodies that protect the eyes from infection. Changes in tear fluid composition, therefore, indicate some pathological abnormalities [9].

1.5.1 Diabetic Retinopathy

Diabetic Retinopathy (DR) is a microvascular complication associated with the abnormal growth of blood vessels on the retina of the eye [4]. One of the biggest public health issues is the diabetes epidemic. Globally, the age-standardized prevalence of diabetes mellitus (DM) has increased by 110% in men and by 58% in women since 1980, reaching 9% and 7.9% respectively in 2014. Combined with population expansion and aging, this frighteningly rapid rate of increase has nearly quadrupled the number of adults with diabetes globally, which is currently estimated to be 422 million and is expected to increase to 629 million by 2045. The majority of DM cases are concentrated in low- and middle-income nations (LMICs) [15]. About 37 million people with DM are blind due to DR. Thus, an increase in patients with related retinal illness is anticipated to coincide with the predicted global DM epidemic [16]. Studies have shown that these diabetes-related complications will cost the USA about $500

million, with a prediction that over 90 million people with diabetes will suffer from DR complications [4].

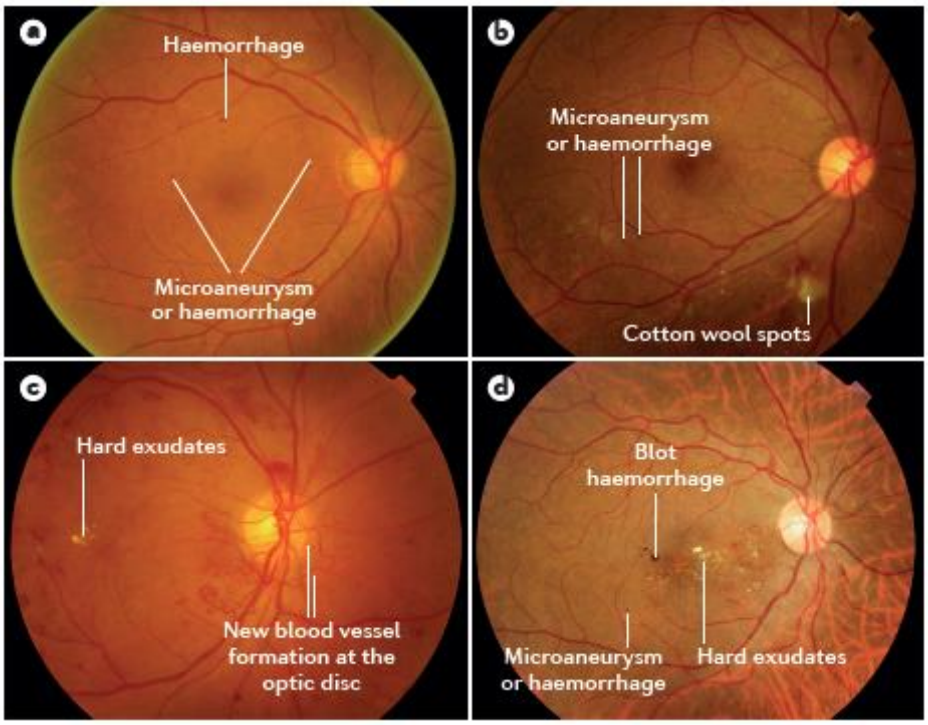


Figure 1. 3: Image of a fundoscopy showing clinical indications of diabetic retinopathy. (a) Mild non-proliferative (b) Moderate non-proliferative DR (c) Proliferative DR exhibiting new blood vessels at the optic disc. (d) Diabetic macular oedema [4].

Diabetic Retinopathy (DR) is a severe complication in both type I and II diabetes that can result in permanent debilitating side effects. The neurological, inflammatory, and microvascular consequence of diabetes referred to as diabetic retinopathy (DR) affects nearly all patients with type I diabetes and has more than 60% prevalence in people living with type II diabetes. DR can permanently cause loss of vision and its risk increases with age [17]. Diabetic retinopathy can be categorized into stages, called non-proliferative (NPDR), and proliferative (PDR) [18]. In some cases, it develops into macular oedema as illustrated in Figure 1.3 [4].

Diabetic Retinopathy is known to be caused by a long history of poor management of blood sugar levels in patients with diabetes. It is determined by the presence of irregular growth of new blood vessels in non-proliferative diabetic retinopathy and proliferative diabetic retinopathy respectively. Chronic hyperglycemia causes metabolic and haemodynamic complications including aggravated vascular permeability, development of microaneurysm,

and death of microvascular cells which gradually spreads around the retina eventually causing vision loss [19]. Several inflammatory responses in DR have been investigated and several biomarkers relating to DR have been identified using tear fluid [12].

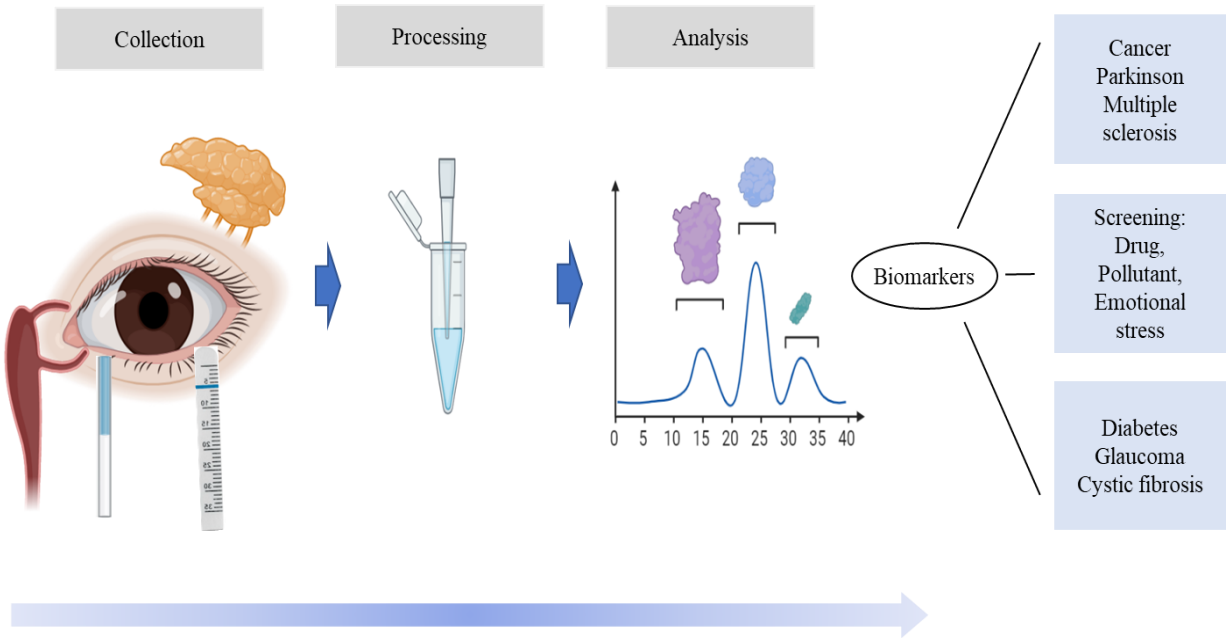


Figure 1. 4: Illustration of clinical methods for tear fluid examination

1.5.2 Tear fluid collection and analysis

The use of tear fluid for early detection of DR provides a crucial means to prevent vision loss in a patient with diabetes mellitus. However, the collection and analysis of tear fluid have been a longstanding challenge for healthcare facilities. While most diagnosis of DR is done by high-end medical devices like fluorescence angiography, optical coherence tomography, or fundoscopy, many healthcare facilities still depend on an experienced ophthalmologist to visually observe the microvasculature morbidity of the retina [1]. This lack of quantitative analysis can result in misdiagnosis of patients causing disease progression and pre-empts effective treatment.

The collection of tear fluid samples has been done in different ways. However, the two most common techniques currently in use are Schirmer strips and capillary tubes [12]. These techniques have varying outcomes in terms of the number of proteins that can be collected [8]. According to some studies, the Schirmer strip technique yields a higher number of

proteins than the capillary tubes technique which yields a low sample amount and requires greater care during the tear collection process. Due to the small sample volume obtainable with these collection methods, various samples from individuals are usually combined to make up enough sample volume before analysis. This results in the loss of individual patient information [14].

The two most popular techniques for collecting tear fluid are the Schirmer strip and the glass microcapillary. One of the clinical methods for collecting samples of tear fluid for examination shown in Figure 3 uses capillary action to collect tear fluid, and the length of a phenol red thread indicates the volume collected in terms of color. After being extracted from the thread, the fluid is next washed and subjected to analysis [12]. Tear fluid presents a beneficial opportunity for researchers to explore a dynamic range of biomarkers from unrelated body parts. Studies have shown the potential use of tears for external screening of drugs and the body's response to drugs. More importantly, the presence of biomarkers in tear fluid has been reported as a valuable source of clinical information in many systemic diseases ranging from ocular diseases including cancer, diabetes mellitus, Parkinson's disease, and Alzheimer's disease [12].

The impact of the collection mode on the composition of tear fluid samples was studied by [8] to analyze a direct comparison between the Schirmer strip technique and the capillary collection technique. The parameters of sampling including the perception of samplers as well as the convenience of participants were assessed. While the collection technique using a capillary tube was considered more convenient, it took samplers more effort to collect tear fluids. The result also shows a significant intra-individual variation found in terms of total protein concentration. This reveals the challenges in maintaining consistency with the existing sample collection methods.

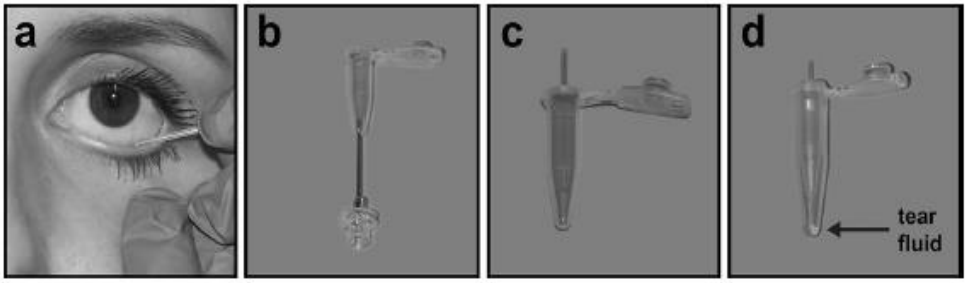


Figure 1. 5: Collecting tear fluid from the lower lid of the eye with 10 µl plastic capillary tubes using capillary action. (a) 10 µl plastic capillary tubes using capillary action from the lower eyelid. (c) Transfer capillary to a punctured 0.5 ml tube (b) and (d) centrifuged retrieved tear fluid after centrifugation [8].

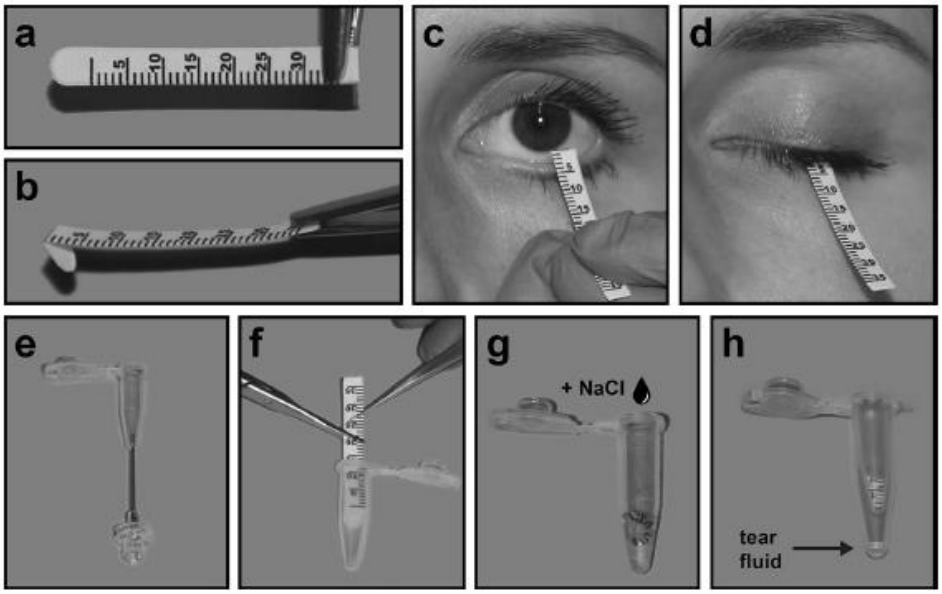


Figure 1. 6: Collecting tear fluid using Schirmer strip [8] (a) Schirmer strip (b) Strip with bent 120° at 0 mark (c) Insertion of the strip for sampling (d) Eye closed for 5min (e) –(f) Soaked strip remove, cut and put into 0.5ml tube (g) 0.9% NaCl added and incubated for 2hrs (h) tube with stirp centrifuged into 1.5ml tube to capture the fluid [8].

1.5.3 Post-Collection Processing of Tear Fluid Samples

The conventional tear fluid screening methods also involve sample storage and laborious preprocessing steps before sample analysis. Denison et al., studied the effect that post-collection processing has on different protein contents of tear fluid obtained using Schirmer strips. In disease diagnosis and treatment, tear fluid provides a potentially abundant source of disease-specific protein biomarkers. However, because of the unpredictability that sample collection and handling procedures introduce, tear fluid sample preparation continues to be a major challenge in tear fluid analysis due to handling losses [20]. This study implicates previous research done on tear analysis but explores other techniques for the use of Schirmer

strip for clinically controlled studies on tear fluids with significant importance. The study established that the collection, storage, extraction, handling, and analysis procedures of tear fluid can have an impact on the composition of the tear fluid sample.

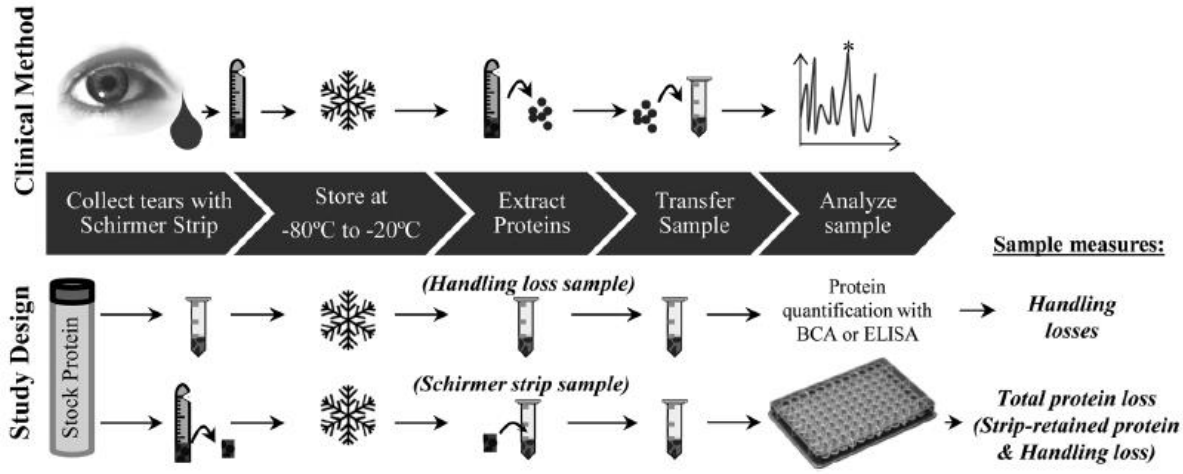


Figure 1. 7: Schematics of existing tear collection and analysis method and experimental setup for isolating two main causes of protein loss: *[20]*.

The sample collection and post-processing technique in Figure 1. 8 shows the *clinical method* and the *study design* processes as illustrated by *[20]*. In the "Study Design" section: A 5 µl sample of protein with a known concentration (referred to as "stock protein") is pipetted into a microtube as the handling loss sample, followed by a 5 µl sample of stock protein loaded onto a 5 mm² Schirmer strip as the Schirmer strip sample and left to dry for 35 minutes at room temperature, and the two samples are then stored overnight at -20°C.

Protein losses that are solely the result of sample manipulation during the elution procedure can be identified and quantified. The entire amount of protein lost as a result of the process is taken into consideration by the Schirmer strip sample since it evaluates the effects of both processes. The study established that the collection, storage, extraction, handling, and analysis procedures of tear fluid can have an impact on the composition of tear fluid.

Contrary to Denison et al., a different approach was proposed by Qin et al., suggesting that tear fluid samples should be conserved together with the record of the patients with a simple, efficient, and low-cost method using vacuum bags to store dry Schirmer strip at room temperature. The study argued that storing tear fluid samples at -80°C is not an effective method of inhibiting protein degradation since the tear fluid samples contain several enzymes and hydrolases. Adding to the already complicated process is the logistical cost of the cold

chain required to transport the sample. A comparative study was done by Qin et al, using a Schirmer strip to collect tear samples from healthy individuals by inserting the strip in the lower eyelids of the healthy study volunteers for 5mins. The strips were then split into two: one for wet storage in the Eppendorf tube at -80°C, and the other for dry storage in a vacuum bag, for 14 days before analysis. Proteins were recovered from stored sample strips and analyzed using a similar process to compare the efficiency of the two methods. Analysis shows a similar confirming the effectiveness of protein preservation by the dry sample storage method proposed [21]. However, this method does not eradicate the subsequent laborious sample processing for analysis and therefore is not ideal for rapid diagnosis.

1.5.4 Diabetic Retinopathy Biomarker in Tear Fluid

The number of people with diabetes is rapidly increasing with no definitive cure in sight. While researchers continue to study its pathogenesis to establish a comprehensive understanding of this disease, better technologies are needed to diagnose and/or predict some of the complications that develop in people with the disease. [17] observed that some level of protein present in the tear fluid of a patient with diabetic retinopathy increases with severity as they age when compared with healthy individuals. Establishing a clear benchmark and fostering research on tear fluid to find diagnostic biomarkers can be achieved by the development of a dependable, quick, and patient-friendly tear fluid collection for speedy diagnosis during routine screening. Although the current and most widely used methods for gathering samples of tear fluid are (Schirmer strips and capillary tubes) considered to be safe and comfortable for patients, significant differences have been identified in the composition and quality of the tear fluid reported so far [8]. Thus, an effective tear fluid analysis technique is needed to reliably diagnose diseases like DR with quicker results in clinical settings.

1.5.5 Current Screening for Diabetic Retinopathy

The gold standard for screening DR is the use of fundoscopy, fluorescein angiography, and optical coherence tomography [4]. However, these technologies are sophisticated, expensive, and require specialist training; therefore they are either not available or not accessible by most patients; hence these available technologies often fall short of meeting the basic needs for effective diagnostics in terms of simplicity, cost-effectiveness, speed, and accuracy [22]. So far, aqueous humor markers, such as inflammatory cytokines and chemokines, have been the focus of the majority of DR biomarker research to date, while

vitreous and plasma have also been studied. These investigations are valuable, but they primarily involve invasive techniques for collecting interior ocular fluids from tissue [16].

There is currently no effective treatment for DR, except laser therapy which is used only in severe cases. This option has been known to cause frequent loss of peripheral vision, color vision, and night vision. However, an early intervention to detect the onset of diabetic retinopathy can prevent the complications of the disease with modifiable risk factors. Therefore, rapid and accurate diagnostic techniques capable of detecting disease biomarkers are needed for timely intervention [18]. Since Tear has an advantage over other body fluids used for screening DR biomarkers due to its non-invasive, quick, and reliable way of collection; better techniques can be developed to resolve the inconsistencies in current tear fluid collection and processing procedures to be able to instantly analyze biomarkers in tear samples.

A different approach to sample collection technique was demonstrated by [12] using an eyeglass-based tear biosensing system that was developed into a wearable tear sampling and analysis system incorporating a microfluidics electrochemical biosensor onto a nose-bridge of an eyeglass. The aim was to non-invasively collect tear fluid samples for instantaneous real-time analysis and monitoring of primary tear biomarkers. Eyeglasses are common accessories used almost daily. Its proximity to tear fluid makes it an important tool for the continuous monitoring of biomarkers. However, This was aimed at addressing some of the limitations associated with other tear fluid analyses using electrochemical biosensor platforms. This idea can be optimized for DR biomarker screening.

1.5.6 Biosensor as a rapid diagnostic tool

Newer developments in bioelectronics have enabled the creation of numerous diagnostic tools and novel methods based on biosensor technology that has proven commercially successful. Biosensors are small, lightweight analytical instruments that can be carried around and can transform a biological response into an analytical signal that can then be amplified, analyzed, and recorded [10]. In contrast to traditional solid-state formats, biorecognition takes place immediately on the surface of a physical sensor. Biosensors are made up of integrated modules that combine the transducer (such as electrochemical, piezoelectric, acoustic, or optical), the detector, and the biological components into a single device. Biosensors, in contrast to traditional bioanalytical systems, do not need additional pre-processing (such as reagents) and are distinguished by the assay design being permanently fixed in the device's architecture [22].

There is a need for efficient point-of-care techniques to help with the growing demands of ocular disease diagnosis in optometric practice [4]. Even while tears have the potential to be a useful source of diagnostic data, challenges with sample collection (a sample size that is too small, as well as sample storage and transit) have shown to be significant constraints. The creation of affordable point-of-care optic fiber sensing technologies is now feasible through advancements in electronics, fiber optics, and sensing technologies. However, a straightforward and sensitive assay approach that would allow one or more significant tear ingredients to be assessed quickly and quantitatively has not been developed, even though the idea of this collecting method is sound and easily adaptable to point-of-care analysis [23]. In addition, The available technologies frequently fall short of achieving the fundamental conditions like simplicity, economy, speed, and accuracy, for a successful diagnosis especially in a resource-constrained setting, even though some technological advancements have matched the standards for implementation in the field [22].

Biosensors that are part of nanobiotechnology have a number of advantages over traditional approaches, including label-free detection, real-time analysis, high-throughput screening, and low limit of detection (LoD). As nanobiotechnology develops quickly, different binding receptors, physicochemical techniques, and nanoplatforms have been utilized, leading to the development of novel tactics for improving detection performance [24]. In contrast to "labeled" biosensors, based on techniques like fluorescence or spectrophotometer, label-free approaches are the main research trend since they enable exact detection at extremely low concentrations without the requirement for any activation or devices external to the sensor itself. Optical biosensors have been presented more recently with the main goal of boosting performances and detection limits by utilizing the high sensitivity of optical methods. Due to their high sensitivity and straightforward construction, biosensors like surface plasmon resonance (SPR) have improved the performance of optical biosensors [25].

Biomarker detection tool is required to have high predictive accuracy, be minimally intrusive, and be straightforward in measurement to be clinically significant. ELISA is the basis for traditional biomarker detection technology used to measure certain protein levels in serum. However, it is reported to have the worst performance in terms of limit of detection (LoD); The results can be influenced by non-specific bindings; it requires a series of complicated operations; and it can only measure one analyte at a time. Consequently, creating new technologies with better capabilities that surpass ELISA can provide better detection opportunities [26].

Optical fiber based-biosensors are a good substitute for easy and affordable diagnostic among the various biosensing platforms because it does not require electrical connections and is not impacted by electric interference like electrochemical biosensors [27]. Biocompatibility, small size, compactness, lightness, resistance to electromagnetic interference, and low production costs are all intrinsic benefits of optical fiber biosensors [10]. Additionally, optical fiber biosensors can be constructed for a multiplexed assay to detection with numerous analytes simultaneously [28].

Harvey et al described a unique method that takes already-existing transferrable technology and ingeniously blends them to produce point-of-care techniques for analyzing electrolytes in individual tear fluid using optical fiber [23]. Since then, a number of technological threads have now come together to make it possible to build low-cost point-of-care fiber optic sensors. Relevant tear analytes of interest may now be measured in clinics instead of in the lab, all due to the flexibility offered by optical fiber and the development of a variety of sensor coatings that can benefit from the combined optics and electronics platform.

Based on the concept proposed by Harvey et al, a scalable low-cost, minimally invasive optical fiber biosensor with a low limit of detection can be developed for use as a point-of-care device for screening key biomarkers in tear fluid of patients with diabetic retinopathy. Earlier research found four main proteins in human tear fluid and lipocalin 1 (LCN-1) protein is one of the highly expressed proteins found in tear fluid of patients with DR with a clinically significant concentration among a control group [1]. Therefore, the incidence of blindness caused by diabetic retinopathy can potentially be minimized significantly using tear fluid as non-invasive sample for screening.

Using optical fiber waveguides for biological analyte detection on the fiber surface allows optical fiber biosensors to achieve great sensitivity and high specificity. These sensors also find significant use as an immunosensors cancer diagnosis, cardiac, and urologic biomarkers detection, as well as in contemporary wearable devices. Refractometers can be functionalized into biosensors to measure the concentration of a particular analyte with improved specificity of detection. Biosensors rely on refractive index (RI) detection as their underlying working principle [29].

1.6 **Proposed** method

The proposed study integrates an optical fiber biosensor in a wearable device for sampling and direct screening of DR biomarkers in tear fluid of diabetes patients. The device will include a microfluidics system driven by a micropump for sample collection; a microcuvette to hold tear fluid collected via a microcapillary tube and an optical fiber ball resonator biosensor positioned into the micro cuvette. This setup eliminates the manual need for tear fluid collection using a Schirmer strip or glass capillary and most importantly avoids laboratory sample pre-processing. With this setup, optical fiber biosensors can also be used in situ to directly interface with the tear fluid towards the lower corner of the eye. Both options will be explored during the research.

This project involves the use of an optical fiber biosensor fabricated from single-mode fiber (SMF) using a CO_2 laser splicing machine (Fujikura LZM-100); and interrogated using optical backscatter reflectometer (OBR 4600, Luna Inc.) to analyze the refractive index (RI). Preliminary fabrication steps will include calibrating the sensors in different concentrations of sucrose solution with stepwise increments to record their refractive index values. Following the RI calibration, the surface of the sensors will be cleaned and functionalized using the silanization method as described in Chapter 2.

CHAPTER 2: Methodology - Laboratory Experiment

2.1 Fabrication of optical fiber biosensor ball resonator

The optical fiber ball resonator was fabricated from single mode standard telecommunication fiber (SMF-28) using Fujikura LZM-100 CO_2 laser splicer Figure 7 (a) to (e). Two strands of standard fiber with a diameter of 125µm were stripped and cleaned with isopropanol to remove the cladding before being loaded on the grooved holders (figure 7 (b)) on the splicer with the set input parameter as shown in **Figure x (c**). A desired diameter of 450µm was set and the distance between the splice point and the center of the ball resonator was set at 1.5mm. The distance between the fibers called the splice offset, was 0.57mm. The rotation speed which enables the creation of a ball tip was set to 100mm/sec.

Figure 2. 1: *Fabrication* of OFBR (a) Fujikura LZM-100 Splicer, (b) inset image of optical fibers layout, (c) Ball resonator Set parameters, (d) and (e) Image of the ball resonator showing x and y diameters.

The Fujikura LZM-100 splicing machine uses high powered CO_2 laser to splice and pull the fiber apart breaking off at the set offset point (0.57mm) while moving the opposite motor with the fiber at 0.025mm/sec. Figure 8 illustrates the fabrication steps of the OFBR by the Fujikura LZM-100.

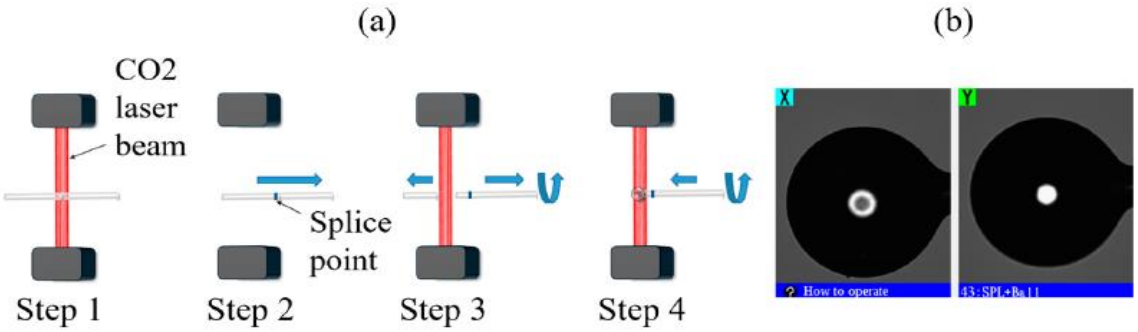
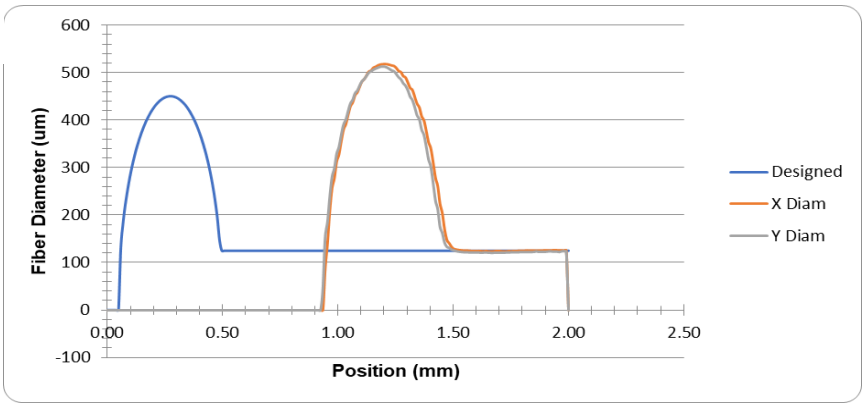


Figure 2. 2: Fabrication process of ball resonator on Fujikura CO2 laser splicer, (b) X and Y diameters view by *a* camera on the splicer *[30]*.

Several optical fiber ball resonators used in this experiment sensors were fabricated in the same way. However, even though the set parameters are the same (Table 1), fabricated OFBR tend to have varying diameters (of the x and y-axis). The difference between the set and estimated horizontal and vertical (x, y-axis) using this method has a low eccentricity error or 7 µm maximum deviation. The misalignment between the ball resonator and the input fiber center was observed by [30] to be minimal (2 µm), and the transition between the input 125 µm SMF and the spherical resonator was constrained to about 40 µm area along the z-axis.

(a)



(b)

Fiber Diameter:	125	**BALL LENS SETTINGS**		Rotation:	TRUE
Ball Diameter:	450	Pre-Heat:	1	Rotator Speed:	100
Diam Adjustment:	-40	Absolute Power:	314	Real-Time Control:	FALSE
Measured X Diam:	518	Relative Power:	170	Feeding Speed:	0.025
Measured Y Diam:	512	Break Add:	20		

Figure 2. 3: Fabrication setup parameters, (a) geometrical profile of fabricated sensor with x and y diameters 529-519µm, (b) Fabrication parameters used.

2.2 Interrogation and refractive index calibration of optical fiber ball resonator

Following the fabrication of the ball resonator, the OBR 4600 optical backscatter reflectometer (OBR 4600, Luna Inc.) was used to interrogate the fabricated optical fiber ball resonator to detect the reflective spectra using the frequency domain between 1527 to 1613nm. The OBR uses the principle of reflectometry within the optical frequency domain with minimal power at the detector to interrogate the ball resonator which acts as a weak interferometer [31]. The ability of the fabricated ball resonator to respond to a change in its surrounding was observed using a varying concentration of sucrose solution with a refractive index. Each OFBR was interrogated as illustrated in **Figure 2.4** by first scanning in the open air, and in PBS before being placed in a 10% sucrose solution. The concentration of the sucrose solution was increased stepwise by adding 400µl of 40% sucrose solution for a total of 5 increments. The RI values were recorded for all concentrations scanned using the OBR 4600.

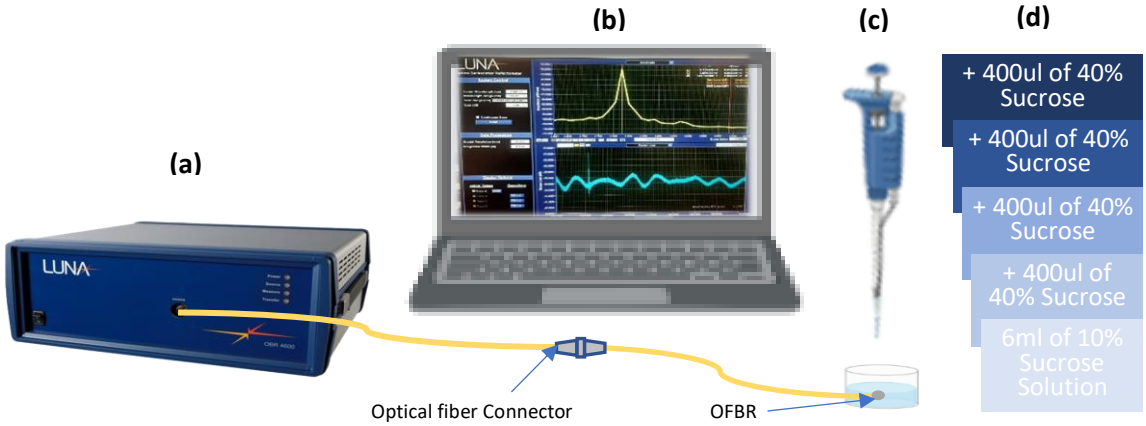


Figure 2.4: Setup for refractive index calibration of the fabricate optical fiber ball resonator. (a) OBR 4600 reflectometer, (b) Laptop, (c) Pipette, (d) Sucrose concentration

The optical fiber ball resonator is fabricated from a telecommunication grade single-mode fiber optic cable (SMF-28) with 125 µm in diameter. It has a higher refractive index (RI) in the core than in its external layer (cladding). The optical ball resonator features a whispering gallery mode (WGM) where light is confined within the internal surface of the sphere (ball resonator). This creates total internal reflection causing light that is near the edge of the ball resonator to be continuously reflected inside the sphere due to the curved interior structure of the ball resonator [32].

The ability of an OFBR to detect a change in its surrounding is assessed by modifying its refractive index in a different medium. The confined internal reflection of the light allows it to interact with the sample/analyte causing Evanescent waves to escape into the environment

[31]. This interaction between the analyte and the surface of the ball resonator results in a change in the refractive index (RI) of the optical field surrounding the ball resonator, which changes the spectral properties of its wavelength and frequency. The changes in RI sensitivity are not specific to a particular detection. However, this indicates the ability of the optical fiber ball resonator to respond to changes around its surface. This makes the ball resonator a relevant tool with several benefits in a variety of biosensing applications.

Since the RI inside the optical ball resonator is higher than the RI of its surrounding, the total internal reflection occurs at the interface of cavity-air contact preventing light from traveling close to the edge of the ball resonator from leaving the sphere. However, there is also a bending loss due to the continuous internal reflection

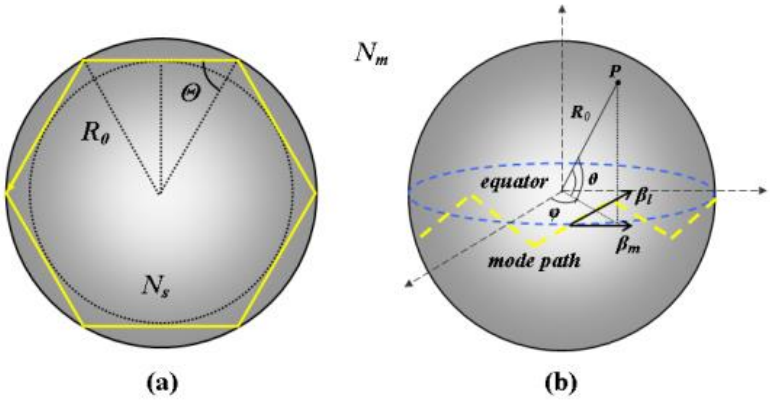


Figure 2.5: (a) Total internal reflection for the light rays in correspondence *with* the surface of the microsphere; (b) Spherical coordinate system and mode propagation along the equatorial plane of the sphere [32].

2.3 Fabrication of a 3D model of the human face

A 3D model of the human face was printed from PLA filament material using Ultimaker^{2+} 3D printer at NU FAB-LAB. The .stl file for the model was obtained from Thingiverse open-source platform (Thingiverse, n.d.). The 3D model was modified to mimic the discharge of tear fluid by the lacrimal gland of the human eye. The right eye of the 3D model was used for the in-situ experimental setup while the left eye of the model was used for the direct tear fluid sampling using flexible capillary tubing with an internal diameter of 0.4 mm. A drain tube was used to assist with the flow of tear fluid as the tear drains through the tear duct.

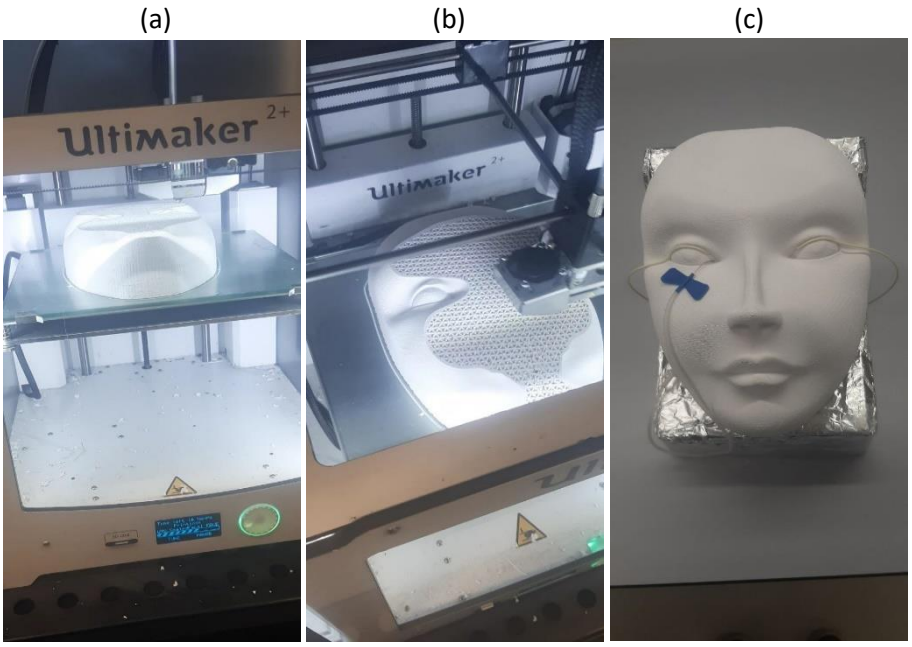


Figure 2.6: 3D printing (a) and (b) of the face model unit *(*Ultimaker^{2+}*)*. (b) *A printed* model with fluidics modification for lacrimal and drain duct.

A wearable eye-glass frame was modified to facilitate the in-situ positioning of the optical fiber biosensor close to the bottom corner of the right eye (the caruncle - between the upper and lower lacrimal puncta). The frame also holds a tear fluid collection system for direct tear fluid sampling for the left eye. The setup on the left eye forms a fluidics system that is connected to a peristaltic pump and tear collection vial. The ball end of the optical fiber biosensor is positioned in the collection vial to directly interface with the collected tear fluid.

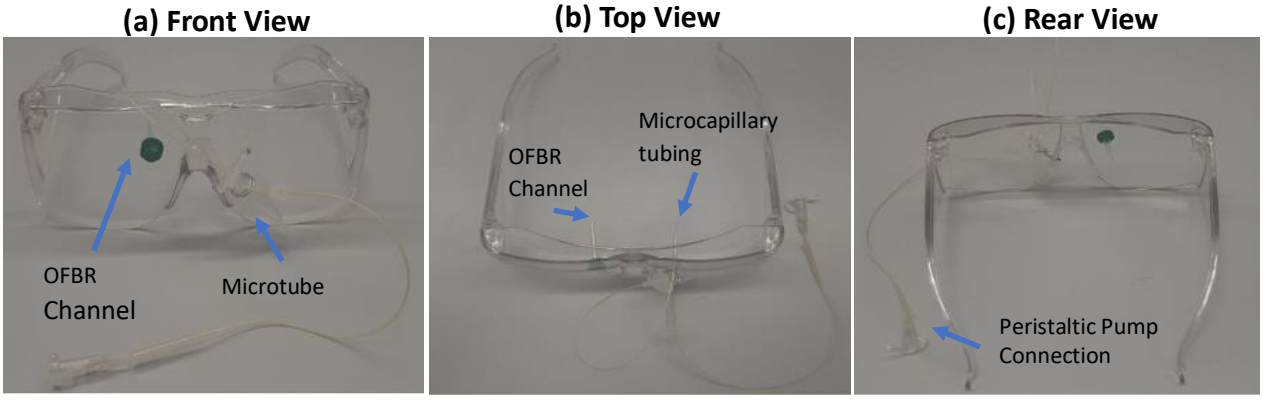


Figure 2.7: Wearable device modified with microtubing for sampling and measurement of biomarkers in tear fluid.

1.6.1 Fabrication of the micro-fluidics on the 3D printed model

This experiment investigates the fabrication of an optical fiber ball resonator, its transformation into a biosensor, and its integration into a device used for the detection of biomarkers in tear fluid. Since tear fluid collection methods influence the quality of the sample as well as the results [33], this experiment aims to evaluate the use of the proposed device to collect tear fluid into a microtube for direct measurement, as well as interface the optical fiber biosensor directly above the lower corner of the eye in contact with tear fluid for analysis.

2.4 Experiment setup for static and dynamic calibration

The setup of the experiment included an optical backscatter reflectometer (Lunar OBR 4600), a laptop with OBR 4600 software for data acquisition and analysis, a syringe pump to generate a predetermined flow of tear fluid, a peristaltic pump, a 3D printed model of the human face with improvised tubing to mimic tear secretion, and a wearable device (eye-goggle as shown in **Figure 2.7** to channel the OFBR to the lower corner of the eye, and ii) to hold the microtube sample collection vial with an OFBR biosensor.

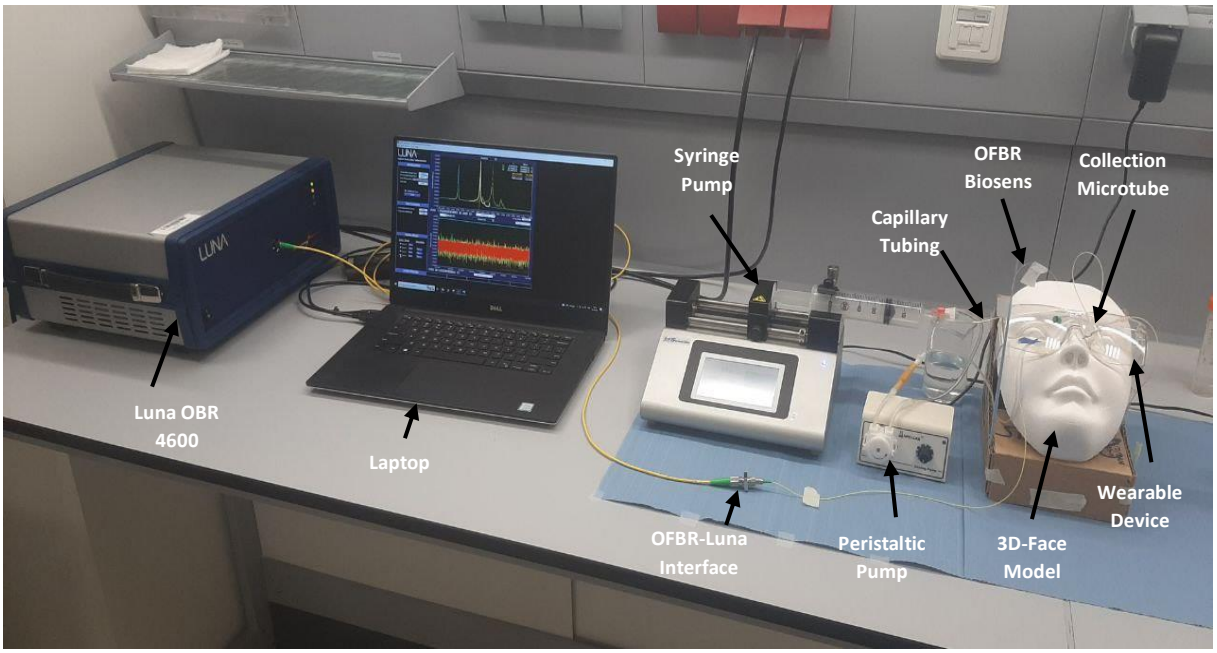


Figure 2.8: Experiment setup for dynamic and static for real-time measurement of LCN-1 biomarker in artificial tear fluid.

2.5 Experiment – Biofunctionalization of OFBR

To target a specific protein of interest, the OFBR was biofunctionalized with antibodies specific to the protein of interest by immobilizing ligands on the surface of the OFBR. The binding of the protein of interest with the functionalized ligand result in a change in the refractive index of the OFBR that varies according to the concentration of the analyte (protein) [25]. This characteristic forms the basis of a labelled-free biosensor.

2.5.1 Surface Functionalization of optical fiber biosensor

To translate the refractive index change of the optical fiber ball resonator into an analysable signal from a biosensor, the surface of the ball resonator was biofunctionalized to facilitate the attachment of ligand (LCN-1 antibody). This was done to convert the changes in RI spectra into a quantifiable optical signal proportional to the concentration of the analyte around the surface of the optical fiber ball resonator. Due to the inert dielectric nature of the optical fiber, one of the two most common methods called silanization was used to functionalize the OFBRs used in this experiment [34]. The process includes cleaning the optical fiber surface, silanization and introduction of cross-linkers, and functionalization with an antibody of interest.

The surface of the fabricated optical fiber ball resonators were functionalized as illustrated in **Figure 2.9** by silanization [35]. The optical fiber ball resonators were labelled according to their diameters (along their x-y axis). Piranha solution was prepared in a fume hood by adding sulfuric acid and hydrogen peroxide in a ratio of 4:1 to make up 30 ml of Piranha solution in a glass tube. The optical fiber ball resonators were placed in the Piranha solution for 15 mins at room temperature to remove organic residues from the surface of the fiber to oxidize the surface. The optical fibers were washed with deionized water and dried with nitrogen gas. A 1% ((3-aminopropyl)trimethoxysilane) APTMS solution was prepared by adding 14.85 ml of methanol to 0.15 ml of APTMS. The optical fibers were placed into the 1% APTMS for 20 mins for the silanization process. The optical fibers were placed in a high-temperature oven for 1 hour at 110 °C to anneal the surface of the optical fiber ball resonator. To add the cross-linker on the surface, the optical fibers were washed with methanol and incubated in 25% Glutaraldehyde (GA) solution and incubated for 1 hour at room temperature. The optical fibers were then washed with PBS before being incubated with 4ul/ml of polyclonal LCN-1 antibody for 1 hour.

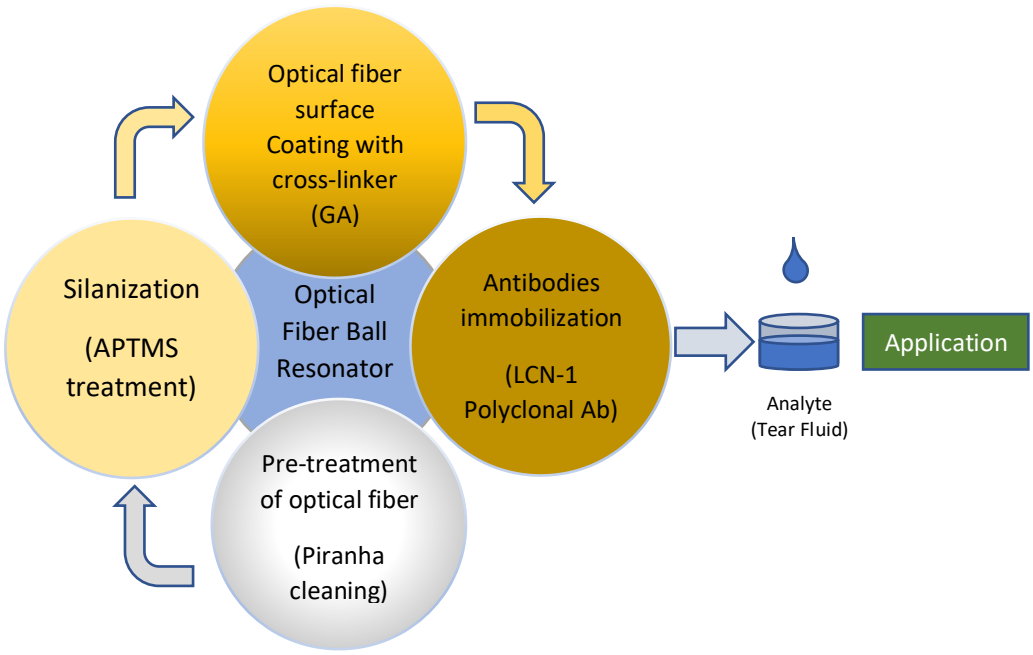


Figure 2. 9: Illustration of the optical fiber biosensor functionalization process.

2.5.2 Evaluation of biofunctionalization processes

The surface changes of OFBR were observed using atomic force microscopy (AFM) after each stage of the biofunctionalization process. This was done to study the quality of the functionalization process based on the density of antibodies used. Bare fibers were stripped and functionalized along with OFBRs. After each stage, one fiber is isolated for imaging with AFM. These stages include Piranha cleaning, Silanization with APTMS, incubation in 25% Glutaraldehyde (GA), attachment of LCN-1 Polyclonal Antibody, and Blocking with mPEG-amine.

2.6 Preparation of artificial tears

An artificial tear solution was prepared according to [33]. The Systane eyedrop (Systane Ultra Plus, Alcon) contains the following ingredients: polyethylene glycol 400, boric acid, calcium chloride, magnesium chloride, potassium chloride, purified water, and sodium chloride). The following ingredient was added according to Moreddu et al.: 5mmol/l of D-(+)-glucose, 120 µmol/l of nitrite solution (from VWR), and 5g/l of protein standard (BSA). The pH of the artificial fluid was measured to be 8.0 using a pH meter (Mettler Toledo).

2.7 Static Detection of LCN-1 Protein in artificial tear fluid

Human recombinant lipocalin 1 (LCN-1) protein (Cloud-Clone, SEB706Hu) was reconstituted with artificial tear fluid. The reconstituted protein was spiked into the artificial tear fluid and diluted in concentrations ranging from 10ng/ml to 1ag/ml in a dilution of the ratio

of 1:10. Several 11 concentrations were prepared (10ng/ml to 0ng/ml). The fabricated and functionalized sensors were used to measure LCN-1 protein spiked in artificial tear fluid using the layout shown in **Figure 2.8.** The OBR 4600 was used as an interrogator operating in the optical frequency domain, along with a laptop, to interpret the spectral change of the biosensor when measuring antibody-protein binding on the surface of the optical fiber biosensor placed in each concentration. An interrogation was made after every 60 seconds and for a total of 10 minutes for every concentration. Two OFBRs were used for measuring LCN-1 protein and their corresponding change in refractive index was recorded.

To study the specificity of the fabricated biosensors, another OFBR was functionalized in the same process as the main biosensors used for the detection of LCN-1 protein (Figure 2.9) and was used to detect CD44 protein spiked in artificial tear with the same concentrations and serial dilution used in the static measurement of the LCN-1. The result obtained is described in Chapter 3.

CHAPTER 3: Results and Discussion

3.1 Sensor fabrication

The physical structure and robustness of the optical fiber ball resonator biosensor were an ideal choice for the proposed concept of integrating a biosensor into a wearable device. In addition, the functionalization technique is also less complex and adaptable. The fabrication of OFBR shown in Figure 2. 10 takes approximately 60 seconds, making it an easily scalable biosensor for mass production. Three OFBRs were used for this experiment. Each OFBR was calibrated in 5 different concentrations of sucrose solution from 10.00 % to 16.32 %.

Table 1: List OFBR used in the experiment

Sensor Diameter	R^2 value	Sensitivity (dB/RIU)	Purpose	Previous Diameter
518-512 µm	0.991481	-89.225165	LCN-1 Dynamic measurement	New
513-506 µm	0.953605	-112.777614	LCN-1 Static measurement	Regenerated from 529-519
443-439 µm	0.955344	-86.464524	Specificity analysis – CD44	Regenerated from 559-531

3.2 Calibration spectrum of OFBRs

The response characteristics of the OFBRs were analyzed using the optical backscatter reflectometer (OBR 4600) during interrogation and calibration as described in Figure 2.11**.** Each OFBR demonstrated some spectral response corresponding to a change in refractive index (RI) and wavelength which is directly proportional to the change in concentration of the analyte around the surface of the sensor. Therefore the concentration of analyte in a biological sample like tear fluid can be measured by analyzing the wavelength shift from the optical fiber biosensor.

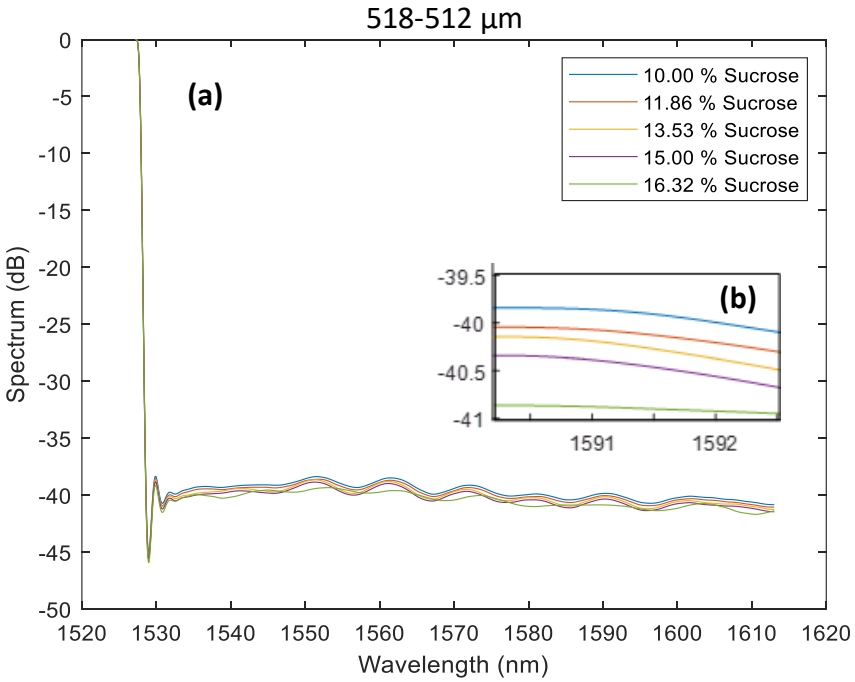


Figure 3. 1: Refractive index (RI) calibration spectrum of OFBR 518-512 µm.

The spectral response of sensor 518-512 µm shown in Figure 3.1 indicates (a) the spectral changes in different concentrations of sucrose solution and (b) the inset of the spectrum at around 1591 nm on the spectrum. This sensor was selected as the main sensor used for the dynamic measurement of LCN-1 in artificial tear fluid using the setup shown in Figure 2.12.

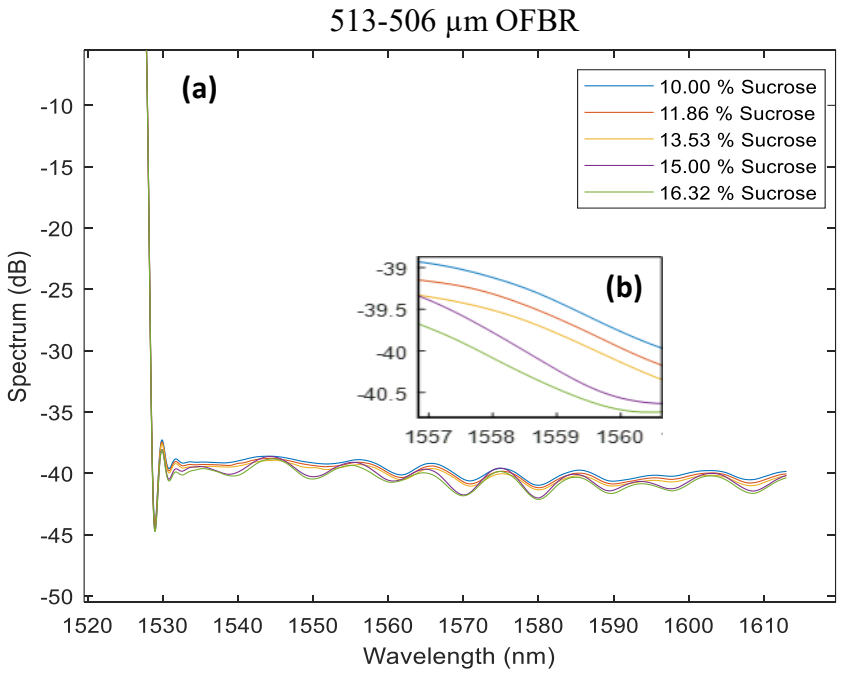


Figure 3. 2: Refractive index (RI) calibration spectrum of OFBR 513-506 µm.

A second OFBR was fabricated to use for the static measurement of LCN-1 in this experiment. Figure 3.1 shows (a) the spectral response of the calibration and (b) an inset of the spectrum at around 1558 nm wavelength.

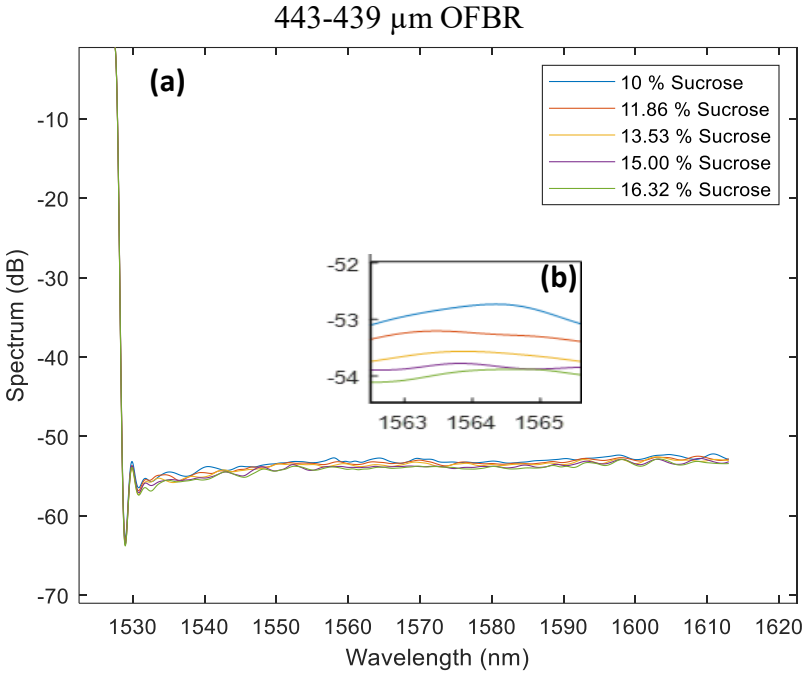


Figure 3. 3: Refractive index (RI) calibration spectrum of OFBR 443-439 µm.

The OFBR 443-435 µm was used for the specificity analysis of the experiment. Figure 3.3 shows its (a) spectral response in varying concentrations of sucrose solutions with (b) inset on the spectrum around 1564 nm wavelength. The spectral response obtained from these OFBRs indicated their ability to respond to be used as biosensors for detecting the concentration of specific biomarkers like LCN-1.

3.3 Biofunctionalization and surface evaluation of OFBRs

All three optical fiber ball resonators were functionalized as described in **Section 2.5.1**. this method has been optimized for use in the ultra-wide attomolar-range detection of CD44 biomarkers [35]. An important aspect of the fabrication of biosensors is the surface functionalization of the optical fiber. The functionalized surfaces of the OFBRs were evaluated using an atomic force microscope (AFM) to study the surface morphology at different stages of the functionalized process. The evaluation shows the surface changes after pre-treatment with Piranha to remove contaminants from the surface activating hydroxyl groups and making the surface more hydrophilic, and silanization with 1 % APTMS to enhance the adhesion layer for further attachment with other ligands and subsequently heated. The silanized surface was

further treated with 25% glutaraldehyde solution to obtain a smooth surface showing similarity with other studies that used the silanization method for as an adhesion layer. The AFM images obtained show qualitative surface morphology after the addition of the antibody (Figure 3.4a). Unreacted aldehyde groups were blocked with mPEG (Figure 3.4b) with height variations across the surface of the optical fibers at the end of the functionalization.

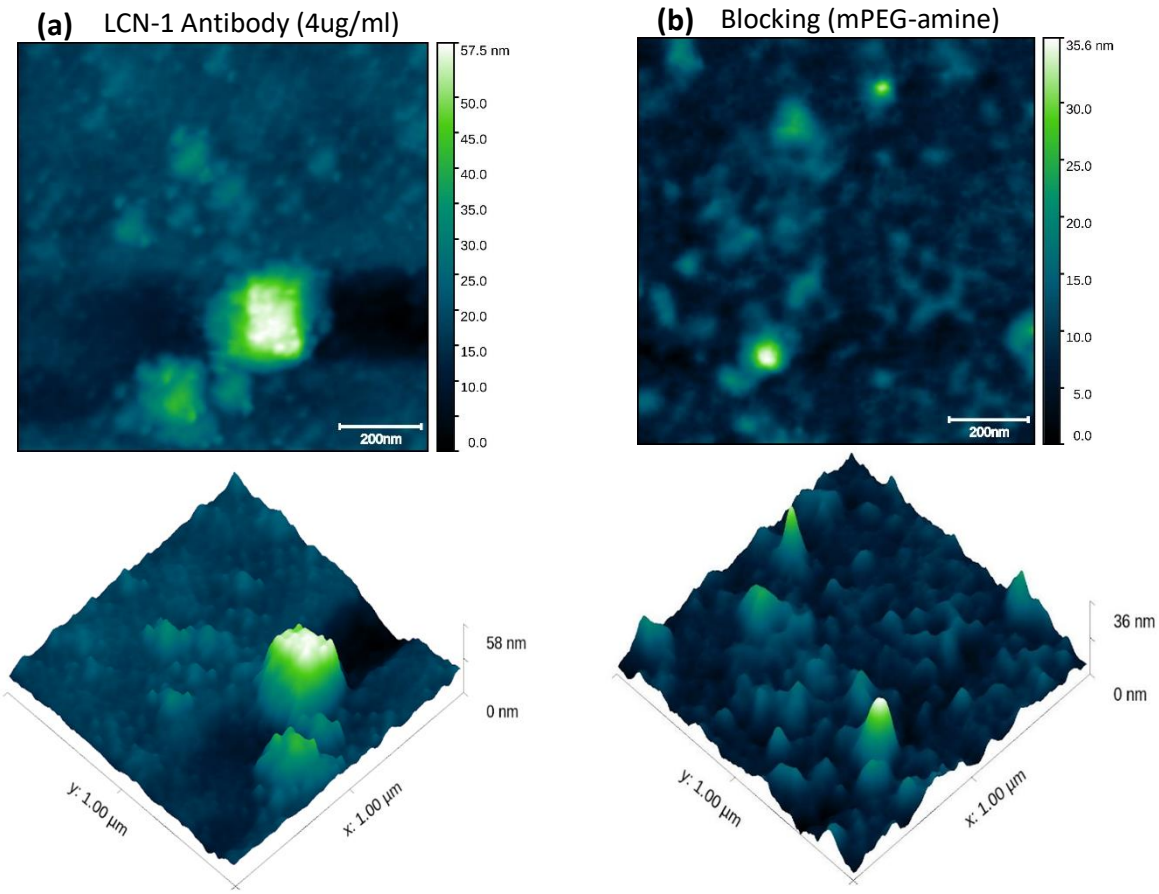


Figure 3. 4: Surface imaging of functionalized biosensor (3D images of AFM) (a) after adding antibody and (b) after blocking with mPEG-amine.

3.4 Static measurement of lcn-1 protein

To the best of our knowledge, The detection of LCN-1 in tear fluid using OFBR has not been done before. This study aims to investigate the ability of OFBR to detect this protein of interest and explore the concept of integrating this biosensor into a wearable device that can be used for screening diabetic retinopathy during a routine eye examination for people at risk of developing diabetic retinopathy. The biofunctionalized OFBR 513-506 µm biosensor was used to detect LCN-1 protein in a static solution of artificial tear. The artificial tear fluid was spiked with LCN-1 protein in a dilution ratio of 1:10 in a serial concentration ranging from 10 ng/ml to 1 ag/ml. This range of concentrations was chosen to first evaluate the characteristics

of the OFBR biosensor in detecting a lower range of LCN-1 while considering the need for detecting higher concentrations in subsequent research experiments.

The setup described in **Figure 1. 9** was developed to enable the static measurement of LCN-1 protein in tear fluid collected in a 0.5 µl tube from the lower corner of the eye. The syringe pump was used to drive the artificial tear fluid through the capillary tubing acting as the lacrimal gland. Artificial tear fluid dispensed on the surface of the eye is aspirated from the lower corner of the eye by a 0.4 mm capillary tubing into the 0.5 µl tube equipped with OFBR biosensor for detection of LCN-1 protein. Using the optical backscatter reflectometer OBR 4600, a total of 10 data points were acquired for each concentration within a duration of 10 mins. Figure 3.5 shows the spectrum of the OFBR 513-506 µm biosensor for the different concentrations during static measurement.

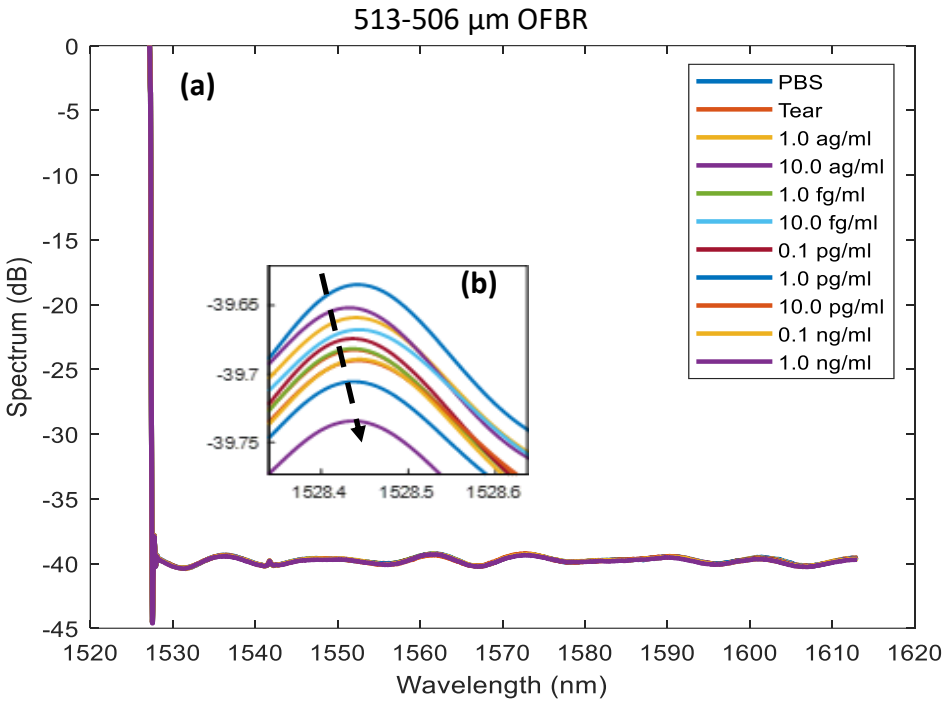


Figure 3. 5: Spectrum of OFBR 513-506 µm biosensor in static measurement showing (a) spectral changes in each concentration and (b) inset on the spectrum around 1528 nm.

3.4.1 Specificity measurement

To determine the specificity of the fabricated biosensors, the OFBR 443-439 µm biosensor functionalized with LCN-1 antibodies was used to detect another protein biomarker that can be found in tear fluid but different from LCN-1. A protein biomarker, CD44 has been reported to be present in human tear fluid and plays several roles including wound healing

[36]. The spectrum obtain shows no significant change in amplitude variation when compared with the response during the static measurement.

The polarization spectrum obtained from the OFBR 513-506 µm shows spectral changes corresponding to the biosensor's response to increasing concentrations of LCN-1 protein in the artificial tear fluid. Figure 3.6 shows the spectrums for the P and S-polarization spectra with increasing concentrations.

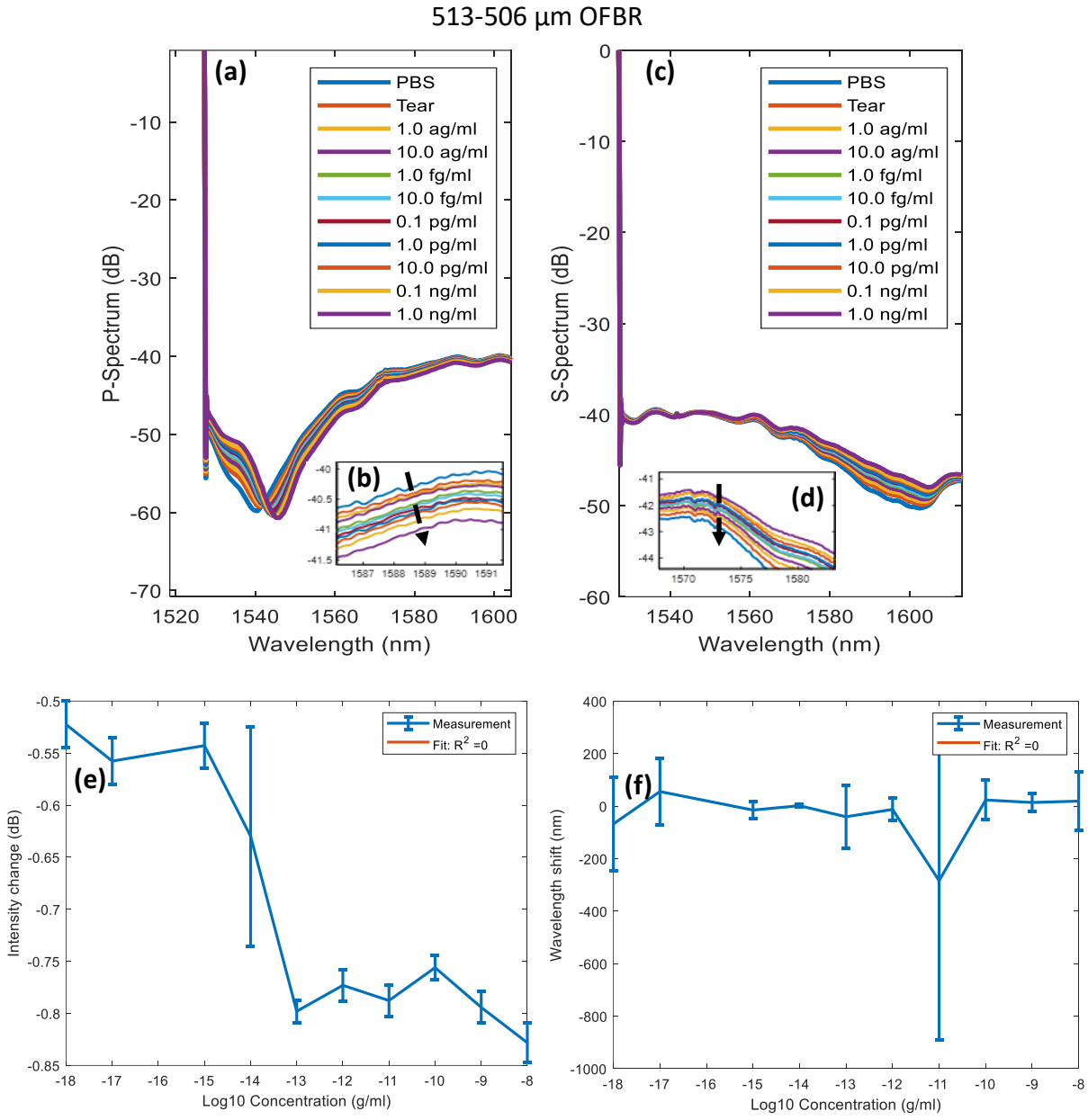


Figure 3. 6: Polarization spectrums of OFBR 513-506 µm: (a) P-spectrum with (b) an inset at around 1600 nm on the spectrum, (c) S-spectrum with (d) inset of around 1546 nm of the polarization in different concentration. (e) the intensity and (f) wavelength with respect to concentration.

The P-spectrum in Figure 3.6(a) shows the OFBR's response to change in intensity corresponding to the binding of the analyte on the surface of the OFBR. This effect causes a change in the refractive index. Hence the spectral change observed in the P-spectrum can be translated to the detection of LCN-1 by the biosensor. While the decrease in intensity observed in Figure 3.6(e) can be due to the non-specific binding of other molecules in the solution since the sensor was functionalized with polyclonal LCN-1 antibody, a decrease in intensity with increasing wavelength was observed in Figure 3.6(f) while the wavelength shift shows no significant change as the concentration increases.

3.5 Dynamic measurement of LCN-1 protein

To demonstrate real-time in-situ measurement according to the proposed concept, the setup described in Figure 2.8 was used to simulate the dynamic flow of tear fluid from the lacrimal glands, over the eye, and to the lacrimal duct for draining. With the wearable device over the 3D printed face model, the OFBR biosensor was inserted through the OFBR channel on the wearable device shown in Figure 2.7(a), towards the lower corner of the eye avoiding direct contact between the tip of the OFBR and the surface tissue of the eye. The OFBR 518-512 µm was used for the dynamic measurement of LCN-1 protein in artificial tear fluid with the same dilution ratio used in the static measurement. Three concentrations were selected from the serial dilution. Using the optical backscatter reflectometer (OBR 4600, Luna), a minimum of 20 measurements were acquired within a duration of 10 minutes. The data obtained were analyzed as shown below using Mathlab software.

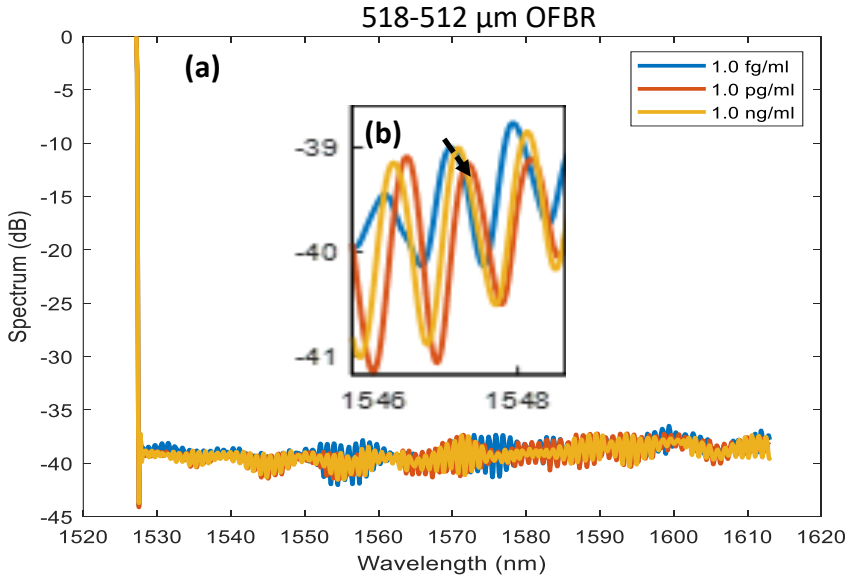


Figure 3. 7: OFBR 518-512 µm spectrum in dynamic measurement of LCN-1.

The spectrum in Figure 3.7(a) shows the spectral response of the biofunctionalized OFBR 518-512 µm biosensor used for in-situ measurement of LCN-1 protein as shown in the experimental setup in Figure 2.8. The inset shown in Figure 3.7(b) indicates a wavelength shift (marked by an arrow) observed around 1548 nm wavelength for the three concentrations in the spectrum.

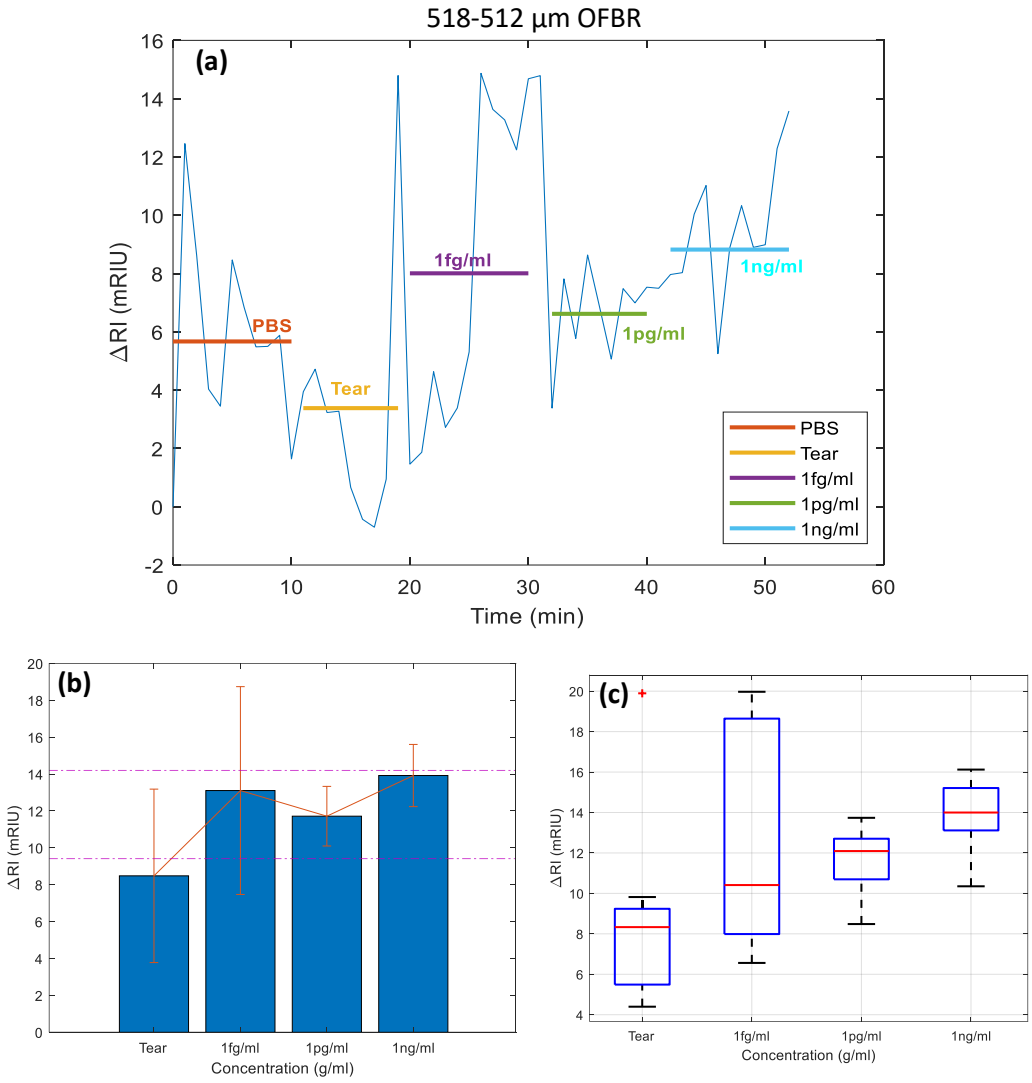


Figure 3. 8: OFBR 518-512 µm biosensor (a) in-situ response with different concentrations of LCN-1 protein. (b) indicate error bars ± standard deviation and (c) BoxPlot distribution

To further evaluate the biosensor's response during the in-situ dynamic measurement, the refractive index change was analysed against the time as shown in Figure 3.8a. The OFBR 518-512 µm was channelled through the wearable device and positioned above the lower corner of the eye, while a syringe pump was used to deliver 500 µl of PBS at a flow rate of about 10 µl/min over the 3D eye model. A minimum of 20 data points were acquired within a duration of 10 mins using the Luna OBR 4600. The same procedure was followed using

artificial tear fluid, and three other concentrations. The trend observed in Figure 3.8a indicates an increase in refractive index (RI) as the concentration increases with time. A significant spike in RI change was observed as the concentration was changed from tear fluid to 1 fg/ml. However, The RI changes in subsequent concentrations show a linear increase with time. The change in refractive index represented in Figure 3.8b shows the error bars with ± standard deviation for tear fluid, 1 fg/ml, 1 pg/ml, and 1ng/ml concentration of LCN-1 during dynamic measurement.

The Boxplot shows differential response during dynamic measurement with the mean values of data for each concentration with an interquartile range of 50% between the third (75^{th}) and first (25^{th}) quartile. The mean values are shown by the red line in each distribution.

CHAPTER 4: Conclusion

This study explores the potentials of optical fiber ball resonator biosensors in terms of shape, miniaturized size and robustness for use in in-situ measurement of biomarkers in body fluids like tears while considering the packaging and commercialization of fabricated optical fiber biosensors. Diagnosing diseases like diabetic retinopathy often require expensive technologies and rigorous sample processing. Hence the need for low-cost devices capable of performing quick diagnostics for early medical intervention.

This experiment, aimed at integrating an optical fiber biosensor onto a wearable device for sampling and direct measurement of LCN-1 biomarkers in tear fluid of people at risk of developing diabetic retinopathy. A low-cost setup was designed to study the proposed concept in real-time for a proof-of-concept and demonstration of its its application. This involved the fabrication of optical fiber ball resonator biosensors that were used for the experiment. The optical fiber ball resonators were biofunctionalized to make them a biosensors for detecting analytes in tear fluid. This setup eliminates the manual need for tear fluid collection using a Schirmer strip or glass capillary and most importantly avoids laboratory sample pre-processing.

The experiment demonstrated that OFBR can detect biomarkers like LCN-1 in tear fluid and in real-time as hypothesized in the proposed concept. However, despite the high performance and technology readiness demonstrated by optical fiber ball resonators, some challenges were registered during the dynamic measurement. This includes signal interference observed due to the reflection of light from the surrounding surface during measurement. Optimizing the setup to accommodate a wider concentration range will also be needed to make the concept relevant in clinical applications. Further experiments using natural tears and an eye model that closely resembles the natural eye could yield better results since the 3D-printed model does not provide the same characteristic. The results obtained from this experiment is promising although further experiments are needed to optimise the biofunctionalization and packaging into a wearable device.

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